characterization of placental bikunin, a novel human serum protease inhibitor

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Katherine A. Delaria‡, Daniel K. Muller§, Christopher W. Marlor‡, James E. Brown¶, Rathindra C. Das‡, Steven O. Roczniak‡, and Paul P. Tamburini**

From ‡Preclinical Research, Biotechnology Unit, §Institute for Bone and Joint Disease and Cancer, and ¶Institute for Research Technologies, Pharmaceuticals Division, Bayer Corporation, West Haven, Connecticut 06516 and **Process Science, Biotechnology Unit, Berkeley, California 94710

We reported previously the cloning of a novel human serum protease inhibitor containing two Kunitz-like domains, designated as placental bikunin, and the subsequent purification of a natural counterpart from human placental tissue (Marlor, C. W., Delaria, K. A., Davis, G., Muller, D. K., Greve, J. M., and Tamburini, P. P. (1997) J. Biol. Chem. 272, 12202–12208). In this report, the 170 residue extracellular domain of placental bikunin (placental bikunin,1–170) was expressed in baculovirus-infected SF9 cells using its putative signal peptide. The resulting 21.3-kDa protein accumulated in the medium with the signal peptide removed and could be highly purified by sequential kallikrein-Sepharose and C₁₈ reverse-phase chromatography. To provide insights as to the potential in vivo functions of this protein, we performed an extensive investigation of the inhibitory properties of recombinant placental bikunin,1–170 and both of its synthetically prepared Kunitz domains. All three proteins inhibited a number of serine proteases involved in the intrinsic pathway of blood coagulation and fibrinolysis. Placental bikunin,1–170 formed inhibitor-protease complexes with a 1:2 stoichiometry and strongly inhibited human plasmin (Kᵢ = 0.1 nM), human tissue kallikrein (Kᵢ = 0.1 nM), human plasma kallikrein (Kᵢ = 0.3 nM) and human factor Xla (Kᵢ = 6 nM). Conversely, this protein was a weaker inhibitor of factor VIIa-tissue factor (Kᵢ = 1.6 μM), factor Xla (Kᵢ = 206 nM), factor Xa (Kᵢ = 364 nM), and factor XIIa (Kᵢ = 430 nM). This specificity profile was to a large extent mimicked, albeit with reduced potency, by the individual Kunitz domains. As predicted from this in vitro specificity profile, recombinant placental bikunin,1–170 prolonged the clotting time in an activated partial thromboplastin time assay.

Blood clotting, resulting either from the extrinsic pathway following tissue injury or the intrinsic pathway following contact activation, involves tightly regulated proteolytic cascades (1). The intrinsic pathway is initiated by activation of factor XII either through proteolysis or contact with negatively charged surfaces. Activated factor XIIa, in turn, converts plasma pro-kallikrein to kallikrein, which can then activate additional factor XII. Factor XIIa activates factor XI, which, in turn, activates factor IX. Activated factor IX forms a complex with factor VIIIa, phospholipid, and calcium, which converts factor X to factor Xa. Factor X is also activated by the factor VIIa-tissue factor factor complex operating within the extrinsic pathway. Thrombin generation by factor Xa in complex with factor Va leads ultimately to the formation of the fibrin clot. Thrombus formation is also regulated by the fibrinolytic system whereby plasmin, formed from plasminogen by the action of kallikrein, tissue plasminogen activator (tPA),¹ or urokinase, breaks down both fibrinogen and fibrin (2).

Protease inhibitors play critical roles in the regulation of the coagulation and fibrinolytic systems. Tissue factor pathway inhibitor (TFPI), a multivalent Kunitz-type inhibitor, is a major regulator of the extrinsic pathway through factor Xa-deendent inhibition of factor VIIa-tissue factor (3). Cl inhibitor is thought to be the major physiological inhibitor of the intrinsic pathway enzymes plasma kallikrein and factor XII (4). More recently, TFPI-2, a serine protease inhibitor structurally related to TFPI, has been proposed to be an important physiological inhibitor of enzymes involved in both coagulation and fibrinolysis (5).

In a previous study, we identified and cloned a human cDNA encoding a novel protein containing two Kunitz-like domains which we termed placental bikunin (6). In this study a soluble recombinant fragment of this protein, placental bikunin,1–170² was expressed and found to be a potent serine protease inhibitor. Using the recombinant protein and both of its synthetic NH₂ and COOH-terminal Kunitz domains, we dissect the specificity of the protein more comprehensively. In doing so, we demonstrate that placental bikunin is a potent inhibitor of plasma and tissue kallikreins, plasmin and factor Xla.

EXPERIMENTAL PROCEDURES

Materials—Bovine chymotrypsin, bovine trypsin, and tPA (single chain form from human melanoma cell culture), urokinase, Suc-A-A-P-F-AMC, Boc-L-G-R-AMC, Suc-A-A-A-AMC, Boc-Q-G-R-AMC, Bz-P-F-R-Ena, and P-F-R-AMC were from Sigma. Neutrophil elastase was from Athens Research and Technology, Inc. (Athens, GA). Human plasmin, ¹The abbreviations used are: tPA, tissue plasminogen activator; TFPI, tissue factor pathway inhibitor; Suc, succinyl; AMC, 7-amido-4-methylcoumarin; Boc, t-butoxycarbonyl; Bz, benzoyl; pNA, p-nitroanilide; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Fmoc, N-(9-fluorenylmethoxycarbonyl); HPLC, high performance liquid chromatography; APTT, activated partial thromboplastin time.

²To whom correspondence should be addressed: Institute for Research Technologies, Bayer Research Center, 400 Morgan La., West Haven, CT 06516-4715. Tel: 203-812-2920; Fax: 203-812-2526; E-mail: pault@wh.bayer.com.

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factor VIIa, tissue factor (lipidated), and (CH$_2$O)$_3$-D-cyclohexylp-rosyl)-G-R-P-R-Pa (Spectrozyme (PA substrate) were from American Diagnostica, Inc. (Greenwich, CT). Human plasma kallikrein and human CoA were from Boehringer Mannheim (Indianapolis, IN). Tos-G-P-AMC, Suc-A-A-P-R-PPa, and Boc-E(OBzl)-R-AMC were from Bachem (Bubendorf, Switzerland. Tos-G-P-AMC, 50 mM Tris-HCl (pH 9.0) for 30 min at room temperature, Bz-P-R-AMC was added (200 μM, final concentration), and residual enzymatic activity monitored at 410 nm. Fractional protease activity was calculated as V$_i$/V$_{-i}$ versus ratio of inhibitor to enzyme concentrations.

**Determination of Equilibrium Dissociation Constants—**Apparent equilibrium dissociation constants ($K_v$) were determined as described previously (6) using methods for tight binding inhibitors (10) assuming enzyme/inhibitor stoichiometries of 1:1 and 2:1 for binding to single Kunitz domains and placentakallikreinit1–170 (1) respectively. Active site concentrations of trypsin, plasma and tissue kallikreins, and plamin (and determined by Bz-P-MR-AMC, with protoporphyrin-p’-guanidino- benzoate as described (11). The concentration of bovine chymotrypsin was determined by titration with N-trans-cinnamoylphenylalalanine as described (12). The amount of the NH$_2$-terminal,7–64, and COOH-terminal102–159 Kunitz domain, and aprotinin were determined by titration with active site-titrated trypsin and by amino acid analysis. The concentration of placentakallikreinit1–170 was quantified by amino acid analysis. The concentrations of factors VIIa, Xa, Xa, Xla, and Xla, tissue factor, elastase, urokinase, and tPA were based on the manufacturer's specifications.

**Determination of Pro tease-Inhibitor Complex—**To determine the stoichiometry with trypsin, the protease (2.2 mM) was incubated with placentakallikrein(0–40 nM) in 1.0 ml of 50 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl and 0.01% Triton X-100 (buffer B). After 30 min at room temperature, Bz-P-R-AMC was added (300 μM, final concentration) and residual enzymatic activity monitored at 410 nm. Fractional protease activity was calculated as V$_i$/V$_{-i}$ versus ratio of inhibitor to enzyme concentrations.

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with 1 mM final (CH₅SO₃)-D-cyclohexylglycylamido-G-RpNa, and the A₄₅₀ nm was monitored. Factor IXa inhibition was measured as described (5). Briefly, factor IXa (10 nM) was preincubated for 15 min at 37 °C with inhibitor in 50 mM Tris-HCl (pH 7.5) containing 0.1 mM NaCl, 250 μM CaCl₂, 60 μM poly-D-lysine (M₀ = 209,200) and 0.1% poly(ethylene glycol) 8000. Factor X (200 nM) was then added and the incubation continued for 30 min at 37 °C. Substrate (600 μM Boc-L-G-Arg-MCA) was then added and factor Xa activity measured. To determine the inhibition of tPA, tPA was preincubated with inhibitor for 2 h at room temperature in 20 mM Tris-HCl (pH 7.2) containing 150 mM NaCl and 0.02% sodium azide. Reactions were initiated with substrate to achieve initial component concentrations of tPA (16.7 nM), inhibitor (0–6.6 μM), 1-P-RpNa (1 mM) in 28 mM Tris-HCl (pH 8.5) containing 0.004% (v/v) Triton X-100 and 0.005% (v/v) sodium azide. The amount of pNa formed after incubation for 2 h at 37 °C was determined from the increase in temperature in the A₄₃₂ nm. Hydrolysis of AMC-conjugated peptides was monitored on a Perkin-Elmer model LS50B fluorometer (excitation = 370 nm, emission = 432 nm) over the first 2 min of the reaction, while hydrolysis of pNa conjugates was monitored at 405 nm on a Hewlett-Packard model HP8452 spectrophotometer. Kᵢ values were determined from plots of fractional rate versus inhibitor concentration, which were fit by nonlinear regression analysis (Enzfit by Biosoft, Cambridge, U. K.) using the following equation

\[ V_{o} = V_{max} \left( \frac{[E]}{[I]_{Ki}} + 1 \right) ^{-1} \left( \frac{[E]}{[I]_{Ki}} + 1 \right) ^{-1} + K_I ^{-1} \left( \frac{[I]}{[I]_{Ki}} + 1 \right) ^{-1} \left( \frac{[E]}{[I]_{Ki}} + 1 \right) ^{-1} \]  

(Eq. 1)

where \( V \) and \( V_{o} \) are the enzyme activities in the presence or absence of a total inhibitor concentration of \([I]_{Ki}\) and \([E]_{Ki}\) is the total concentration of enzyme. \( K_I \) values were obtained by correction for the effect of substrate using the following equation: \( K_I = K_I^{o} / (1 + [S]/K_c) \). \( K_c \) values for inhibition by placental bikunin were determined assuming an inhibitor:protease stoichiometry of 1:2 and that the two Kunitz inhibitory domains are equivalent and act independently.

Measurement of the Activated Partial Thromboplastin Time (APTT)—The effect of recombinant placental bikunin, and aprotinin on the APTT was determined as follows. Inhibitor in 20 mM Tris-HCl (pH 7.2) containing 150 mM NaCl and 0.02% sodium azide was added (0.1 ml) to a cuvette within a MLA Electra 8000 Automatic Coagulation Timer coagulometer (Medical Laboratory Automation, Inc., Pleasantville, NY). The instrument was set to APTT mode with a reaction volume of 60 μl, a spray voltage, 5 kV; capillary temperature, 220 °C; nitrogen pressure, 50 p.s.i.; auxiliary nitrogen, 30 ml/min. Samples were loaded on a PLRP C18 (5 μm) column, washed with water, and eluted at 0.2 ml/min with 90% (v/v) acetonitrile, 2% (v/v) acetic acid.

RESULTS

To explore the protease inhibitory capacity of placental bikunin, a cDNA encoding the entire extracellular domain of bikunin on the APTT was determined as follows. Inhibitor in 20 mM Tris-HCl (pH 7.2) containing 150 mM NaCl and 0.02% sodium azide was added (0.1 ml) to a cuvette within a MLA Electra 8000 Automatic Coagulation Timer coagulometer (Medical Laboratory Automation, Inc., Pleasantville, NY). The instrument was set to APTT mode with a reaction volume of 60 μl, a spray voltage, 5 kV; capillary temperature, 220 °C; nitrogen pressure, 50 p.s.i.; auxiliary nitrogen, 30 ml/min. Samples were loaded on a PLRP C18 (5 μm) column, washed with water, and eluted at 0.2 ml/min with 90% (v/v) acetonitrile, 2% (v/v) acetic acid.

Characterization of Human Placental Bikunin

170 amino acids of placental bikunin plus its natural signal peptide (6) was prepared by site-directed mutagenesis and expressed in the baculovirus/Sf9 system. The protein was purified from the cell culture supernatant by sequential kallikrein-Sepharose affinity chromatography and C₁₈ reverse-phase HPLC (Table I). An overall enrichment of 240-fold was achieved based on the increase in specific activity; however, the actual fold enrichment of the protein was likely to be much higher as only a fraction of the inhibitory activity of the culture supernatant was due to placental bikunin. This latter conclusion is based on the fact that the majority (>99%) of the trypsin inhibitory activity present in the starting supernatant did not bind to the kallikrein-Sepharose column, whereas all the placental bikunin bound the column based on immunoanalysis of the column starting material and flow-through (not shown). Fractionation of 2 liters of medium typically yielded between 0.7 and 1 mg of placental bikunin based on amino acid analysis.

The final preparation was highly pure as judged by SDS-polyacrylamide gel electrophoresis (Fig. 1) and exhibited a molecular mass of 21.3 kDa, consistent with the expected size of 20 kDa. NH₂-terminal sequence analysis of the protein (26 cycles) yielded the same NH₂ terminus for as obtained for the natural protein (6). The sequence started at residue +1 and continued with the sequence ADGER-. Furthermore, the amino acid composition was >95% accurate relative to the theoretical composition of placental bikunin. Purified placental bikunin (100 pmol) was then pyridylethyl alkylated, CNBr digested, and then sequenced without purification of the resulting peptide fragments. Sequencing for 20 cycles yielded the sequence for each of the NH₂ termini expected from the digestion of placental bikunin (Table II).

Titration of fixed concentrations of either bovine trypsin or human plasma kallikrein with purified placental bikunin showed that each molecule of inhibitor can interact with two molecules of protease simultaneously (Fig. 2). On the other hand, titration of trypsin with aprotinin yielded a stoichiometry of 1:1 in the enzyme inhibitor complex (Fig. 2).

To attempt to dissect the specificities of the individual domains within this bikunin, synthetic peptides were made corresponding to the NH₂-terminal, COOH-terminal, and Kunitz domains of placental bikunin. Purified, reduced NH₂-terminal (1-64) (M[H – H] = 5,647.5, and COOH-terminal (102-159) domain (M[H – H] = 6,835.6) were prepared, each of which had the expected amino acid composition. Refolding of these peptides using dimethyl sulfoxide as the oxidizing agent, followed by purification on a C₁₈ column, yielded purified refolded NH₂-terminal (1-64) (2% of the starting reduced peptide) and COOH-terminal (102-159) domains (1.4% of the starting reduced peptide) which exhibited [M-H]⁺ values of 6,561.2 and 6,829.3, respectively. The mass reduction upon refolding of each peptide (6 ± 1 mass units) suggests that refolding had in each case resulted in the formation of intrachain disulfide bonds from the six cysteines present within each of the reduced peptides.

A panel of serine proteases was used to determine the
Characterization of Human Placental Bikunin

The individual Kunitz domains within placental bikunin were each functional protease inhibitors and exhibited roughly similar specificities, although the NH₂-terminal(7–64) Kunitz domain was significantly less potent against tissue kallikrein and factor Xa. Only versus factor XIIa was the NH₂-terminal domain more potent than the COOH-terminal domain. Overall, the specificity of the COOH-terminal Kunitz(102–159) domain more closely resembled that of recombinant placental bikunin, except that on average the potency of the COOH-terminal Kunitz(102–159) for each protease was approximately 5-fold lower.

**DISCUSSION**

In this report we describe the expression and functional characterization of placental bikunin. Because the natural protein was available only in low amounts (6) the functional studies were performed instead with a recombinant form of the protein. A cDNA sequence encoding a 170-residue placental bikunin fragment containing both Kunitz domains but truncated immediately prior to the putative transmembrane segment (6) was expressed in Sf9 cells. This was done to alleviate concerns that expression of the full-length protein would complicate its recovery as a soluble protein. The putative signal peptide encoded within the cloned cDNA (6) was recognized as such by the Sf9 system as judged by its absence from the NH₂-terminal sequence of the purified protein. For comparison with the recombinant protein, the individual Kunitz domains were prepared synthetically and effectively folded as judged by reductions in mass consistent with formation of the expected number of intrachain disulfide bonds. Recombinant placental bikunin, and the synthetic NH₂-terminal(7–64) and COOH-terminal(102–159) Kunitz domains were all potent serine protease inhibitors. Each protein was a potent inhibitor of trypsin, chymotrypsin, plasmin, factor Xa, and tissue and plasma kallikrein, yet a relatively weak inhibitor of factors IXa, Xa, and XIIa. Stoichiometry studies with placental bikunin verified that both Kunitz domains were functional and could direct the formation of ternary complexes with

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**Table II**

<table>
<thead>
<tr>
<th>Digestion was performed according to “Experimental Procedures.”</th>
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<tr>
<td><strong>Sequence</strong></td>
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<td>----------------</td>
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<tr>
<td>LRCF–QQENPP–PLG</td>
</tr>
<tr>
<td>ADRERS1HDPCV6SKVVGRC</td>
</tr>
<tr>
<td>FNYeVCYTAHVSPCRASP</td>
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</table>

* Lowercase letters denote tentative amino acid assignments.

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**Fig. 1.** SDS-polyacrylamide gel electrophoresis analysis of placental bikunin, highly purified from a baculovirus/Sf9 expression system. Three μg of highly purified placental bikunin (lane 2) and molecular size markers of the indicated sizes in kDa (lane 1) were resolved on a 10–20% Tricine-SDS-polyacrylamide gel and developed with Coomassie Blue.

**Fig. 2.** Stoichiometry of complex formation between placental bikunin and serine proteases. Increasing concentrations of placental bikunin were incubated with a fixed concentration of either trypsin or kallikrein under stoichiometric conditions followed by determination of residual protease activity. Titration of trypsin with aprotinin is shown for comparison. Stoichiometries are obtained from the x axis intercept of the tangent to curves passing through the y axis intercept.
suggests that this inhibitor could function to stabilize fibrin versus time of human plasma by placental bikunin (1–170) and aprotinin. The high potency of the other hand, the kallikreins play a key upstream role in contact activation and the intrinsic pathway of coagulation. On that might be expected of a physiological regulator of both findings, placental bikunin exhibits an activity profile that might be expected of a physiological regulator of both contact activation and the intrinsic pathway of coagulation. Based on these findings, placental bikunin exhibits an in vitro activity profile that might be expected of a physiological regulator of both contact activation and the intrinsic pathway of coagulation. The potent of the entire extrinsic pathway terminates. The possibility that placental bikunin may regulate the intrinsic pathway through a similar cooperative mechanism warrants further investigation.

We have demonstrated that although the NH2- and COOH-terminal Kunitz domains could reflect the fact that the putative N-linked carbohydrate residue within the NH2-terminal domain of native placental bikunin (6) plays a role in determining the specificity of this domain.

In general, placental bikunin(1–170) exhibited K values that were significantly lower than those observed with the individual domains. Similar results have been obtained with TFPI wherein the recombinant protein containing all three of the Kunitz domain had anywhere from a 2-fold to 10-fold greater affinity for the factor VII-tissue factor complex than any of the individual domains (15). Furthermore, cooperatively has been demonstrated for the interaction of TFPI with its target proteases. Specifically, Girard and Broze demonstrated that TFPI inhibits the tissue factor-factor VIIa complex 50-fold more potently when first allowed to complex with factor Xa (16). From a biological perspective this is consistent with a feedback inhibition mechanism allowing some active factor Xa to form before the entire extrinsic pathway terminates. The possibility that placental bikunin may regulate the intrinsic pathway through a similar cooperative mechanism warrants further investigation. We have demonstrated that although the NH2- and COOH-terminal Kunitz domains have similar potencies versus a number of proteases, the COOH-terminal Kunitz domain is 10-fold more potent against factor Xla. Perhaps the COOH-terminal domain of placental bikunin inhibits factor Xla in vivo, which in turn triggers the subsequent formation of a ternary complex through interaction of the NH2-terminal domain with factor XIIa or kallikrein. This would serve as a feedback inhibition mechanism for the intrinsic pathway where some factor Xla is allowed to form before the entire pathway is shut down. On the other hand, the differences in potency against factor Xla between the synthetic NH2- and COOH-terminal Kunitz domains could reflect the fact that the putative N-linked carbohydrate residue within the NH2-terminal domain of native placental bikunin (6) plays a role in determining the specificity of this domain.

Blockage of fibrinolysis and extracorporeal coagulation have been observed in open heart surgery patients receiving aprotinin (17, 18), and these effects are believed to arise from the inhibition of kallikrein, plasmin, and the intrinsic pathway of coagulation (19). Because placental bikunin(1–170) is significantly more potent than aprotinin as an inhibitor of plasma kallikrein, and the intrinsic pathway of coagulation, further exploration of the use of this novel human bikunin in this

<table>
<thead>
<tr>
<th>Pro tease</th>
<th>NH2-terminal 1–64 Kunitz domain</th>
<th>COOH-terminal 102–159 Kunitz domain</th>
<th>Placental bikunin(1–170)*</th>
<th>Aprotinin</th>
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</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>0.03 ± 0.01 (13)</td>
<td>0.05 ± 0.01 (13)</td>
<td>0.01 ± 0.002 (12)</td>
<td>0.02 ± 0.003 (13)</td>
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<td>Chymotrypsin</td>
<td>1.7 ± 0.54 (11)</td>
<td>2.1 ± 0.2 (12)</td>
<td>0.48 ± 0.15 (11)</td>
<td>1.3 ± 0.43 (8)</td>
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<td>Plasma kallikrein</td>
<td>0.57 ± 0.14 (8)</td>
<td>0.73 ± 0.09 (11)</td>
<td>0.31 ± 0.04 (9)</td>
<td>13.4 ± 2.4 (11)</td>
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<td>Pancreatic kallikrein</td>
<td>0.42 ± 0.09 (11)</td>
<td>0.49 ± 0.13 (11)</td>
<td>0.28 ± 0.06 (10)</td>
<td>0.023 ± 0.006 (6)</td>
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<td>Tissue kallikrein</td>
<td>2.3 ± 0.5 (10)</td>
<td>0.13 ± 0.02 (7)</td>
<td>0.13 ± 0.03 (10)</td>
<td>0.004 ± 0.003 (7)</td>
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<tr>
<td>Plasmin</td>
<td>1.04 ± 0.25 (11)</td>
<td>0.54 ± 0.11 (9)</td>
<td>0.10 ± 0.03 (9)</td>
<td>0.18 ± 0.06 (11)</td>
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<tr>
<td>Factor IXa/Xa*</td>
<td>N.I. (2 µM)</td>
<td>507 ± 15 (3)</td>
<td>206 ± 14 (6)</td>
<td>N.I. (5 µM)</td>
</tr>
<tr>
<td>Factor Xa</td>
<td>274 ± 60 (7)</td>
<td>364 ± 97 (8)</td>
<td>N.I. (10 µM)</td>
<td>N.I. (30 µM)</td>
</tr>
<tr>
<td>Factor Xla</td>
<td>107 ± 25 (7)</td>
<td>15 ± 38 (9)</td>
<td>5.9 ± 1.4 (11)</td>
<td>298 ± 71 (6)</td>
</tr>
<tr>
<td>Factor XIIa</td>
<td>477 ± 140 (6)</td>
<td>800 ± 245 (6)</td>
<td>429 ± 134 (6)</td>
<td>6800 ± 2,840 (7)</td>
</tr>
<tr>
<td>FVIIa-tissue factor*</td>
<td>N.D.</td>
<td>N.D.</td>
<td>1606 ± 264</td>
<td>N.I. (1 µM)</td>
</tr>
</tbody>
</table>

* K values for placental bikunin(1–170) were determined by nonlinear regression analysis assuming [I]0 equal to twice the molar protein concentration.

Enzymes from bovine sources.
Enzymes from human sources.
Apparent equilibrium dissociation constant.
N.I., no inhibition at the highest inhibitor concentration tested (value in parentheses).
N.D., not determined.
clinical setting is warranted. In addition, placental bikunin should also be considered for other indications for which aprotinin has been shown to be beneficial preclinically. One potential application is the blockage of the plasmin-dependent invasion of cancer cells into surrounding tissue (20). In addition, placental bikunin may be useful for the reduction of severity of brain edema secondary to brain or spinal chord injury (21, 22), a process that may result from the local release of the vasodilator bradykinin (21–23) through the actions of serine proteases such as kallikrein (24).

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